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In re Patent Application of:
Nigel Atherton et al.

Application No.: 09/891,206 Confirmation No.: 9337

Filed: June 26, 2001 Art Unit: 1616

For: TREATMENT OF BONE DISEASES Examiner: J. D. Pak

SUBMISSION OF DOCUMENTS

Commissioner for Patents
P.O. Box 1450
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Dear Sir:

Applicant claimed priority upon filing this application with the USPTO under 35 U.S.C. 119 to the following prior foreign application filed in the following foreign country on the date indicated:

Country	Application No.	Date
United Kingdom	0015745.3	June 27, 2000

In support of this claim, a certified copy of the said original foreign application is filed herewith.

Dated: April 27, 2005

Respectfully submitted,

By _____
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27 JUN 2000

1. **Your reference** 40.41.72647

2. **Patent application number** *(The Patent Office will fill in this part)* **0015745.3**

28JUN00 E548463-1 D00027
P01/7700 0.00-0015745.3

3. **Full name, address and postcode of the or of each applicant** *(underline all surnames)*

Shire Holdings AG
Bundersstrasse 5
6300 Zug
Switzerland

Patents ADP number *(if you know it)* 79297 3000/

If the applicant is a corporate body, give country/state of incorporation Switzerland

4. **Title of the invention** Treatment of bone diseases

5. **Name of your agent** *(if you have one)* Frank B. Dehn & Co.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

179 Queen Victoria Street
London
EC4V 4EL

Patents ADP number *(if you know it)* 166001

6. **If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and** *(if you know it)* **the or each application number**

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. **If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application**

Number of earlier application	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

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12. Name and daytime telephone number of person to contact in the United Kingdom

Derek Woodman
020 7206 0600

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Treatment of bone diseases

This invention relates to the treatment and prevention of bone diseases, and also to the treatment of bone fracture.

Throughout life, old bone is continuously removed by bone-resorbing osteoclasts and replaced with new bone which is formed by osteoblasts. This cycle is called the bone-remodelling cycle and is normally highly regulated, i.e. the functioning of osteoclasts and osteoblasts is such that the same amount of bone is formed as is resorbed.

The bone-remodelling cycle occurs at particular areas on the surfaces of bones. Osteoclasts which are formed from appropriate precursor cells within bones resorb portions of bone; new bone is then generated by osteoblastic activity.

Irregularities in one or more stages of the bone-remodelling cycle (e.g. where the balance between bone formation and resorption is lost) can lead to metabolic bone diseases. Examples of such diseases are osteoporosis, Paget's disease and rickets. Some of these diseases are caused by over-activity of one half of the bone-remodelling cycle compared with the other, i.e. by osteoclasts or osteoblasts. As an example, osteoporosis is a disease in which a relative increase in osteoclastic activity causes a reduction in bone density and mass. Osteoporosis is the most common of the metabolic bone diseases and may be either a primary disease or may be secondary to another disease or other diseases.

Post-menopausal osteoporosis is currently the most common form of osteoporosis. Senile osteoporosis afflicts elderly patients of either sex and younger individuals occasionally suffer from osteoporosis.

Osteoporosis is characterised generally by thinning and weakening of bones leading to increased fracturing from minimal trauma. The most prevalent fracturing in post-menopausal osteoporotics is of the wrist and spine. Senile osteoporosis, is characterised by a higher than average fracturing of the femur.

Whilst osteoporosis as a therapeutic target has been of and continues to attract a great deal of interest, tight coupling between the osteoblastic and osteoclastic activities of the bone remodelling cycle make the replacement of bone already lost an extremely difficult challenge. Consequently, research into treatments for prevention or prophylaxis of osteoporosis (as opposed to replacement of already-lost bone) has yielded greater results to date.

Oestrogen deficiency has been considered to be a major cause of post-menopausal osteoporosis. Indeed steroids including oestrogen have been used as therapeutic agents (*New Eng. J. Med.*, **303**, 1195 (1980)). However, recent studies have concluded that other causes must exist (*J. Cain. Invest.*, **77**, 1487 (1986)).

Other bone diseases can be caused by an irregularity in the bone-remodelling cycle whereby both increased bone resorption and increased bone formation occur. Paget's disease is one such example.

The present invention is based on the surprising finding that lanthanum compounds have beneficial effects on the activity and differentiation of bone cells, as discussed in more detail below.

According to one aspect of the invention, there is provided a method for management, treatment or prophylaxis of bone disease or management or treatment of bone fracture which comprises administering to a human or animal subject suffering from, or susceptible to bone disease a therapeutically or prophylactically effective amount of a lanthanum compound.

In this text, "susceptible to bone disease" is

intended to embrace a higher than average predisposition towards developing bone disease. As an example, those susceptible towards osteoporosis include post-menopausal women, elderly males (e.g. those over the age of 65) and those being treated with drugs known to cause osteoporosis as a side-effect (e.g. steroid-induced osteoporosis).

According to a further aspect of the invention there is provided the use of a lanthanum compound for the preparation of a medicament for use in the management, treatment or prophylaxis of bone disease, or in the management or treatment of bone fracture.

According to a still further aspect of the invention there is provided the use of a lanthanum compound for the preparation of a pharmaceutical composition for use in the diagnosis of bone disease or of bone fracture.

The term "lanthanum compound" is used herein to denote any pharmacologically acceptable lanthanum compound capable of ensuring that the lanthanum is bioavailable. Preferred compounds include, for example, lanthanum salts and derivatives thereof, lanthanum resins and lanthanum absorbants. The lanthanum may if desired be in the form of a chelate. Hereinafter, the invention will be described with specific reference to certain lanthanum salts and derivatives.

Hereinafter, references to treatment of bone diseases are intended to include management and/or prophylaxis except where the context demands otherwise.

A wide variety of bone diseases, may be treated in accordance with the present invention, for example all those bone diseases connected with the bone-remodelling cycle. Examples of such diseases include all forms of osteoporosis, osteomalacia, rickets and Paget's disease. Osteoporosis, especially of the post-menopausal, male and steroid-induced types, is of particular note. In addition, lanthanum compounds find use as antiresorption

agents generally, as bone promotion agents and as anabolic bone agents. Such uses form another aspect of the present invention.

The lanthanum compounds of the invention may be administered in the form of a pharmaceutical composition comprising the active ingredient in admixture or association with a pharmaceutically acceptable carrier or diluent. The active ingredient may be formulated into a composition suitable for administration by any convenient route, e.g. orally (including sublingually), topically, parenterally (including intravenous, intramuscular, intraperitoneal and subcutaneous administration) and rectally, oral administration being preferred. It should be understood, however, that the invention embraces all forms of administration which make the lanthanum systemically available.

Orally administrable compositions may, if desired, contain one or more physiologically compatible carriers and/or excipients and may be solid or liquid. The compositions may take any convenient form including, for example, tablets, coated tablets, capsules, lozenges, aqueous or oily suspensions, solutions, emulsions, syrups, elixirs and dry products suitable for reconstitution with water or another suitable liquid vehicle before use. The compositions may advantageously be prepared in dosage unit form. Tablets and capsules according to the invention may, if desired, contain conventional ingredients such as binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth or polyvinyl-pyrollidone; fillers, for example lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; lubricants, for example magnesium stearate, talc, polyethylene glycol or silica; disintegrants, for example potato starch; or acceptable wetting agents such as sodium lauryl sulphate. Tablets may be coated according to methods well known in the art.

Liquid compositions may contain conventional

additives such as suspending agents, for example sorbitol syrup, methyl cellulose, glucose/sugar syrup, gelatin, hydroxymethylcellulose, carboxymethylcellulose, aluminium stearate gel or hydrogenated edible fats; emulsifying agents, for example lecithin, sorbitan monooleate or acacia; non-aqueous vehicles, which may include edible oils, for example vegetable oils such as arachis oil, almond oil, fractionated coconut oil, fish-liver oils, oily esters such as polysorbate 80, propylene glycol, or ethyl alcohol; and preservatives, for example methyl or propyl *p*-hydroxybenzoates or sorbic acid. Liquid compositions may conveniently be encapsulated in, for example, gelatin to give a product in dosage unit form.

Compositions for parenteral administration may be formulated using an injectable liquid carrier such as sterile pyrogen-free water, sterile peroxide-free ethyl oleate, dehydrated alcohol or propylene glycol or a dehydrated alcohol/propylene glycol mixture, and may be injected intravenously, intraperitoneally, subcutaneously or intramuscularly.

Compositions for rectal administration may be formulated using a conventional suppository base such as cocoa butter or another glyceride.

Compositions for topical administration include ointments, creams, gels, lotions, shampoos, paints, powders (including spray powders), pessaries, tampons, sprays, dips, aerosols, pour-ons and drops. The active ingredient may, for example, be formulated in a hydrophilic or hydrophobic base as appropriate.

It may be advantageous to incorporate an antioxidant, for example ascorbic acid, butylated hydroxyanisole or hydroquinone in the compositions of the invention to enhance their storage life.

Administration in this invention may consist of one or more cycles; during these cycles one or more periods of osteoclastic and osteoblastic activity will occur, as

well as one or more periods when there is neither osteoclastic nor osteoblastic activity.

Alternatively, administration may be conducted in an uninterrupted regimen; such a regimen may be a long term regimen, e.g. a permanent regimen.

It will be understood that the dosages of compositions and the duration of administration according to the invention will vary depending on the requirements of the particular subject. The precise dosage regime will be determined by the attending physician or veterinary surgeon who will, *inter alia*, consider factors such as body weight, age and symptoms (if any). The compositions may if desired incorporate one or more further active ingredients.

If desired, the lanthanum compound may be administered simultaneously or sequentially with other active ingredients. These active ingredients may, for example include other medicaments or compositions capable of interacting with the bone remodelling cycle and/or which are of use in fracture repair. Such medicaments or compositions may, for example, be those of use in the treatment of osteoarthritis or osteoporosis.

Figures 1 to 4 show the effect of the lanthanum (III) ion on bone resorption, osteoclast differentiation, osteoblast differentiation and bone formation respectively.

The following passages describe the effect of a particular lanthanum (III) ion-containing solution using *in vitro* bone culture assays.

Test substance

The test substance was lanthanum carbonate tetrahydrate (hereinafter lanthanum carbonate). 1 mg of lanthanum is equivalent to 1.9077 mg of lanthanum carbonate. Lanthanum carbonate was dissolved in 2M HCl to give a

concentration of 28.6 mg/ml (i.e 15 mg/ml of lanthanum). Aliquots of this stock solution were diluted with 2M HCl to result in solutions of varying concentrations, so that addition of one microliter of these solutions into the culture medium gave the final test concentrations of 100, 500, 1000, 5000 and 15000 ng/ml of lanthanum in culture medium. These solutions/concentrations are hereinafter referred to as LA100, LA500, LA1000, LA5000 and LA15000.

Control substances

We used control groups in each assays to show that the assays were capable of detecting the effect of inhibition (bone resorption assay and osteoclast differentiation assay) or activation (osteoblast differentiation and bone formation). The control substances used were:

- Bafilomycin A1 (in bone resorption assay)
- 17-β estradiol (in osteoblast differentiation assay and bone formation assay)

In the osteoclast differentiation assay, the control group did not contain vitamin D.

Bone resorption assay

The method of osteoclast culture on bone slices was originally described by Boyde *et al.* (1984) and by Chambers *et al.* (1984). For cell culture, we used a method slightly modified from the original methods (Lakkakorpi *et al.* 1989, Lakkakorpi and Väänänen, 1991). The rate of bone resorption in the cultures was originally determined by counting the number of resorption pits on each bone or dentine slice using a microscope with phase contrast objectives (Sundquist *et*

al. 1990). Later, the pits were visualized using Wheat Germ Agglutinin lectin that specifically binds to the resorbed area in bone (Selander *et al.* 1994), making it possible to quantify the total resorbed area using a microscope and computer-assisted image analysis system (Laitala and Väänänen 1994, Hentunen *et al.* 1995). We used a commercially available method (CrossLaps for cultures, Osteometer Biotech, Herlev, Denmark) to detect the amount of collagen cross-links released into the culture medium as an index of the bone resorption rate (Bagger *et al.*, 1999).

The study protocol uses a method where osteoclasts are cultured on bone slices and allowed to resorb bone. The system is ideal for determining the effect of drug candidates on the bone resorbing activity of osteoclasts. Drug candidates are added into the cell cultures at the beginning of the culture period, and the osteoclasts allowed to resorb bone for 3 days. The amount of bone resorbed during the culture period is determined and compared to the amount of bone resorbed in control cultures (those cultured in the absence of drug candidates). If the drug candidate inhibits the function of osteoclasts, the amount of bone resorbed in these cultures is significantly lower than in the control cultures.

References:

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Hentunen TA, Lakkakorpi PT, Tuukkanen J, Lehenkari PP, Sampath TK,Väänänen BK (1995) Effects of recombinant human osteogenic protein-1 on the differentiation of osteoclast-like cells and bone resorption. Biochem Biophys Res Commun 209: 433-443.

Laitala T, Väänänen HK (1994) Inhibition of bone resorption *in vitro* by antisense RNA and DNA molecules targeted against carbonic anhydrase II or two subunits of vacuolar H+-ATPase. J Clin Invest 93: 2311-2318.

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Osteoclast differentiation assay

A method known as mouse bone marrow culture system is the one most widely used to study osteoclast differentiation. Originally, this method was developed by Takahashi *et al.* (1988a). Osteoclast precursors in mouse bone marrow can be induced to form multinucleated osteoclast-like cells (MNC) in the presence of either an active metabolite of vitamin D_3 ($1,25(OH)_2D_3$) or parathyroid hormone (PTH). MNC formed in mouse bone marrow cultures have been demonstrated to possess several features characteristic of osteoclasts. They form pits on bone or dentine slices (Takahashi *et al.* 1988a, Hattersley and Chambers 1989, Shinar *et al.* 1990); they express high levels of tartrate-resistant

acid phosphatase (TRAP) and calcitonin receptors (Takahashi *et al.* 1988b, Shinar *et al.* 1990); and they respond to calcitonin (Takahashi *et al.* 1988a) and prostaglandin E_2 (Collins and Chambers 1992). Thus, the method is an ideal one with which to study both stimulators and inhibitors of osteoclast differentiation.

In the original culture system, the osteoclast formation was determined after an 8-day culture. In bone marrow, both non-adherent osteoclast precursors and stromal cells are present, the latter of which are needed to support osteoclast formation. The number of osteoclasts formed is generally determined by counting the number of TRAP-positive MNC containing at least three nuclei (Takahashi *et al.* 1988a). In the negative control, where $1,25(OH)_2D_3$ is not added, TRAP-positive MNC are not formed.

We have modified the original assay so that we culture 1×10^6 mouse marrow cells/ml for 6 days. With this modification, the number of TRAP-positive MNC/culture has been shown to be approximately 150 (Choi *et al.* 1998, Hentunen *et al.* 1998). Instead of counting of the number of differentiated osteoclasts formed, we measured the amount of TRAP liberated from osteoclasts into the culture medium using a fast, simple TRAP immunoassay (Patent pending; Halleen *et al.* 1999). This method has been presented in the Annual Meeting of the American Society for Bone and Mineral Research, September 30 — October 4, 1999, in St. Louis, MO, USA. Our results show that the amount of TRAP released into the culture medium correlates significantly ($r = 0.94$, $p < 0.0001$, $n = 120$) with the amount of osteoclasts formed.

References

Halleen N, Alatalo S, Hentunen TA, Väänänen HK (1999) A novel TRAP 5b immunoassay for osteoclast cultures. J Bone Miner Res 14, Suppl. 1, S244.

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Hattersley G, Chambers TJ (1989) Generation of osteoclastic function in mouse bone marrow cultures: multinuclearity and tartrate-resistant acid phosphatase are unreliable markers for ostcoclastic differentiation. Endocrinology 124: 1689-1696.

Hentunen TA, Reddy SV. Boyce BF, Dovlin R, Park H-R, Chimg H, Selander K S, Dallas M, Kurihara N, Galson OL, Goldring SR, Koop, BA Windle JJ, Roodman GD (1998) Immortalization of osteoclast precursors by targeting bcl-X_L and simian virus 40 large T antigen to the osteoclast lineage in transgenic mice. J Clin Invest 102: 88-97.

Shinar DM, Sato M, Rodan GA (1990) The effect of hemopoietic growth factors on the generation of osteoclast-like cells in mouse bone marrow cultures. Endocrinology 126: 1728-1735.

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Takahashi N. Akatsu T, Sasaki T, Nicholson GC, Moseley JM, Martin TJ, Suda T (1988b) Induction of calcitonin receptors by lα,25-dihydroxyvitamin D_3 in osteoclast-like multinucleated cells formed from mouse bone marrow cells. Endocrinology 123: 1504-1510.

Osteoblast differentiation assay

Osteoblasts are bone-forming cells which arise from mesenchymal stem cells. During the development of osteoblasts, three distinct periods have been identified and defined: 1) cell proliferation and secretion of extracellular matrix (ECM); 2) ECM maturation; and 3) ECM mineralization. During these periods, a sequential

expression of osteoblast phenotype markers has been characterized. Alkaline phosphatase is associated with the bone cell phenotype and is actively expressed during the maturation of the osteoblast. With the onset of mineralization, large amounts of calcium are deposited into the mature organic matrix to form bone-like nodules. By following these markers, we are able to study all the stages of osteoblast differentiation in this culture system.

Several methods have been devised to study osteoblasts. The first of these involves isolation of cells from calvaria with the osteoblastic phenotype. However, these cells only represent the mature stage of osteoblasts, because only a small fraction of the calvarial cells are osteoblast precursors (Bellows and Aubin 1989, Bellows *et al.* 1994). Osteoblastic cell lines are convenient in use, but they may not behave as primary osteoblasts (Mundy 1995). It is conceivable that osteoblast precursors are present in bone marrow (Friedenstein 1976, Owen 1988), and bone marrow stromal cells have long been recognized as the source of osteoprogenitor cells.

We have established a culture model in which mouse bone marrow derived osteoprogenitor cells first proliferate and then differentiate to osteoblasts capable of forming mineralized bone nodules (Qu *et al.* 1998, Qu *et al.* 1999). We confirmed this by following the expression of several markers of the osteoblastic phenotype and by studying the morphology of cultures at light and electron microscopic level. Synthesis of fibrillar extracellular matrix with late deposition of calcium confirmed the differentiation and maturation of osteoblasts. Thus, this culture system fulfills requirements of an *in vitro* model useful for studying differentiation of osteoprogenitor cells into bone

synthesizing osteoblasts.

References

Bellows CG, Aubin JE (1989) Determination of the number of osteoprogenitors in isolated fetal rat calvarial cells in vitro. Develop Biol 113:8-13.

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Qu Q, Härkönen PL, Väänänen HK (1999) Comparative effects of estrogen and antiestrogens on differentiation of osteoblasts in mouse bone marrow culture. J Cell Biochem 73: 500-507.

Bone formation assay

The activity of mature osteoblasts can be determined by quantifying their ability to form mineralized bone matrix. This is done by demineralizing the formed bone matrix, and determining the amount of calcium released. Thus, this culture system fulfills requirements of an *in vitro* model useful for studying the bone formation activity of mature osteoblasts.

References

Bellows CG, Aubin JE (1989) Determination of the number of osteoprogenitors in isolated fetal rat calvarial cells in vitro. Dev Biol 113:8-13.

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Qu Q, Perälä-Heape M, Kapanen A, Dahllund J, Salo J, Väänänen HK, Harkönen, P (1998) Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. Bone 22:201-209.

Qa Q, Harkonen PL, Väänänen HK (1999) Comparative effects of oestrogen and antiestrogens on differentiation of osteoblasts in mouse bone marrow culture. J Cell Biochem 73: 500-507.

Animals

Species/strain/age/sex	Supplier
Mouse/NMRI< 8-12 w, male and female	University of Turku, The centre of experimental animals, Turku, Finland
Rat, Sprague-Dawley, 1 day	University of Turku, The centre of experimental animals, Turku, Finland

PROCEDURES

Bone resorption assay

Transverse 0.1 mm thick slices of cortical bone were cut from the diaphysis of fresh bovine femurs (Atria Slaughterhouse, Oulu, Finland) using a low-speed diamond saw, cleaned by ultrasonication in multiple changes of sterile distilled water, and stored at 4°C before use. Long bones were removed from 1-day-old rat pups killed by decapitation. The bones were dissected free of adherent soft tissues, and the endosteal surfaces were curetted with a scalpel blade into the osteoclast culture medium (Dulbecco's Modified Eagle's Medium (DMIEM), (Gibco BRL, Paisley, UK)) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin (Penicillin/Streptomycin solution, Gibco BRL, Paisley, UK), 20 mM HEPES buffer (Gibco BRL, Paisley, UK) and 10% heat-inactivated fetal calf serum, pH 6.9 (Gibco BRL, Paisley, UK). The resulting suspension of dispersed cells and bone fragments was agitated using a plastic pipette. Larger fragments were allowed to sediment for a few seconds and the supernatant was seeded onto the bone slices pre-wetted in the medium. After a settling period of 30 minutes at 37°C, the bone slices were washed by dipping in fresh medium, and then transferred to wells in 24-well culture dishes containing osteoclast culture medium. The bone slices were incubated in a humidified atmosphere of 95 % air and 5 % carbon dioxide at 37°C for 72 hours.

After the culture period, the amount of bone resorption was determined by measuring the amount of collagen cross-links released into the culture medium using a commercial kit (CrossLaps for cultures, Osteometer Biotech) according to the manufacturer's instructions. The number of osteoclasts in each culture was determined by microscopic counting of the amount of TRAP-positive multinuclear cells, and the results are given as the number of collagen cross-links released per one osteoclast.

In this study, the effect of the lanthanum (III) ion on the bone resorbing activity of osteoclasts was tested.

The following sample groups were included:

- Baseline (including vehicle)
- Control (Baseline + 10 nM bafilomycin A1)
- Baseline + 100 ng/ml lanthanum
- Baseline + 500 ng/ml lanthanum
- Baseline + 1000 ng/ml lanthanum
- Baseline + 5000 ng/ml lanthanum
- Baseline + 15000 ng/ml lanthanum

Six replicates were included in each group, and the test was performed twice. Bafilomycin A1, a highly potent inhibitor of osteoclast V-ATPase proton pump, was used as a control to show the ability of the test system to detect inhibition of bone resorption.

Osteoclast differentiation assay

8-10-week old mice were killed with CO_2. Tibia and femora were dissected free from adhering soft tissues. The bone ends were cut off with a scalpel and the marrow was flushed with α-Minimal Essential Medium (α-MEM, Gibco BRL, Paisley, UK) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. A 10ml syringe with a 27 gauge needle was used for flushing. Cells were centrifuged at 600 x G for 10 minutes and the cell pellet was resuspended in α-MEM containing 10% fetal calf serum. Cells were allowed to attach to plastic for 2 h at 37 °C in a 5% CO_2 incubator to allow removal of monocytes and macrophages. Nonadherent cells were duly removed, and the attached bone marrow cells were cultured in 24-well plates (1 x 10^6 cells/well = 1 ml) for 6 days. Half of the media were changed at day 3 and

the treatments replaced. At the end of the culture, the plates were fixed with 2 % paraformaldehyde in PBS for 20 minutes. Osteoclast formation was determined by measuring TRAP activity from the culture media using the novel TRAP immunoassay (*vide infra*), where we use a polyclonal TRAP antiserum prepared in rabbits against purified human bone TRAP. The TRAP antibody was bound to anti-rabbit IgG coated microtiter wells (Gibco BRL, Paisley, UK), and medium TRAP was then bound to the antibody. The activity of bound TRAP was measured in sodium acetate buffer using pNPP as substrate.

In this study, the effect of the lanthanum (III) ion on osteoclast differentiation in the presence of 1,25-dihydroxyvitamin D3 was tested. The following sample groups were included:

- Baseline (including vehicle)
- Control (Baseline without 1,25-dihydroxyvitamin D3)
- Baseline + 100 ng/ml lanthanum
- Baseline + 500 ng/ml lanthanum
- Baseline + 1000 ng/ml lanthanum
- Baseline + 5000 ng/ml lanthanum
- Baseline + 15000 ng/ml lanthanum

Six replicates were included in each group, and the test was performed twice. Baseline without 1,25-dihydroxyvitamin D3 was used as a control to show the test system allows inhibition of osteoclast differentiation to be detected. As the results of LA100 did not give statistically the same result (significantly different or not compared with the baseline) in both of the two tests, we performed the test with LA100 one additional time.

Osteoblast differentiation assay

Bone marrow cells were obtained from the femurs of 10-week old female NMRI mice. Animals were killed by cervical dislocation. Both femora were removed and the soft tissues were detached aseptically. Metaphyses from both ends were cut off and bone marrow cells were collected by flushing the diaphysis with culture medium: phenol red-free-α-modified essential medium (α-MEM (Gibco BRL, Paisley, UK)). A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a 22 gauge needle, and nucleated cells were counted with a hemocytometer. Cells were plated at 10^6 cells/cm^2 in T-75 tissue culture flasks in phenol red-free α-MEM supplemented with 10 % FCS, 10^{-8} M dexamethasone, 50 µg/ml ascorbic acid, 10^{-2} M sodium β-glycerophosphate, 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were cultured for 6 days and half of the media replaced after 3 days. On day 6, subcultures were prepared. Cells were washed with warm PBS and adherent cells were detached using trypsin-EDTA. Trypsinized cells were passed through a syringe with a 22 gauge needle to make a single-cell suspension, counted and plated in 24-well plates at a density of 5×10^3 cells/ml. These osteoprogenitor cells were stimulated to differentiate towards mature osteoblasts by culturing them in the presence of 10^{-10} M estrogen (17β-estradiol) for 8 days. The test substances were added at the beginning of the secondary culture without estrogen, and every time when the medium was changed.

The number of osteoblasts formed was determined by measuring cellular alkaline phosphatase (ALP) activity in the culture. Cells were disrupted by washing the cell layers twice with PBS, extracting into 200 µl 0.1 % Triton X-100 buffer at pH 7.6 (Sigma, St. Louis, MO, USA), and overnight freezing. ALP activity was determined colorimetrically using *p*-nitrophenylphosphate

as substrate at pH 9.7 and determining the optical density at 405 nm. In parallel, protein contents of the wells were determined by the BIO-RAD protein assay, and the specific ALP activity is expressed as units/mg protein.

In this study, the effect of the lanthanum (III) ion on osteoblast differentiation was tested. The following sample groups were included:

- Baseline (+ vehicle)
- Control (Baseline + 10^{-10} M 17β-estradiol)
- Baseline + 100 ng/ml lanthanum
- Baseline + 500 ng/ml lanthanum
- Baseline + 1000 ng/ml lanthanum
- Baseline + 5000 ng/ml lanthanum
- Baseline + 15000 ng/ml lanthanum

Bone formation assay

The mature osteoblasts obtained during the 8-day secondary culture in the absence of estrogen and any test substances described above were allowed to form bone nodules by culturing them for 7 additional days. At the end of the culture, the amount of calcium deposited during the culture period was determined, and the amount of bone formation (calcium deposition) calculated.

In order to quantify the amount of calcium deposited, the cell cultures were washed three times with Ca^{2+}- and Mg^{2+}-free PBS and incubated overnight at room temperature in 0.6M HCl. Extracts of 50 µl were complexed with 1 ml determined *o*-cresol-phthalein-complexon. The colorimetric reaction was determined at 570 nm in a spectrophotometer. Absolute calcium concentrations were determined by comparison with a calibrated standard provided by the vendor.

In this study, the effect of lanthanum carbonate on bone formation was tested. The following sample groups were included:

- Baseline (including vehicle)
- Control (Baseline + 10^{-10}M 17β-estradiol)
- Baseline + 100 ng/ml lanthanum
- Baseline + 500 ng/ml lanthanum
- Baseline + 1000 ng/ml lanthanum
- Baseline + 5000 ng/ml lanthanum
- Baseline + 15000 ng/ml lanthanum

Statistical analyses

The mean and standard deviation (SD) of each group was determined. One-way analysis of variance (ANOVA) was used to study if the values obtained between different groups (baseline vs. controls and test substances) were statistically different (with $p < 0.05$). Statistical significance is shown in each table and figure with asterisks, one asterisk (*) indicating a p-value between 0.05 and 0.01, two asterisks (**) a p-value between 0.01 and 0.001, and three asterisks (***) a p-value K 0.001. No asterisks indicate that the results of the group do not differ significantly from the results of the corresponding baseline group.

RESULTS

Bone resorption assay

In the bone resorption assay, the amount of medium CrossLaps (nM) released into the culture medium was determined and the number of osteoclasts in the corresponding cultures calculated. The medium CrossLaps

amounts were divided with the osteoclast numbers in the corresponding cultures, and the results are given on Table 1 as relative medium CrossLaps amounts per osteoclasts. The relative values were obtained by dividing each individual value with the mean value of the baseline group.

Table 1: Relative medium CrossLaps amounts per osteoclast in the first bone resorption assay

Group	1	2	3	4	5	6	Mean ± SD
Baseline	0.98	0.82	1.01	1.65	0.74	0.81	1.00±0.34
Control	0.00	0.00	0.00	0.19	0.27	0.14	0.10±0.11(***)
LA 100	0.57	0.56	1.13	0.78	0.71	0.71	0.74±0.21
LA 500	1.04	0.58	1.38	0.75	0.88	0.63	0.88±0.30
LA 1000	1.14	1.09	0.89	1.76	1.07	1.11	1.18±0.30
LA 5000	1.39	0.78	2.70	1.18	0.76	1.21	1.34±0.71
LA 15000	0.57	0.58	0.57	0.96	2.53	1.11	1.05±0.76

Table 2: Relative medium CrossLaps amounts per osteoclasts in the second bone resorption assay

Group	1	2	3	4	5	6	Mean ± SD
Baseline	0.75	1.33	0.88	1.98	0.53	0.53	1.00±0.56
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00±0.00(***)
LA 100	0.38	0.75	0.78	0.94	0.67	0.96	0.74±0.21
LA 500	0.50	2.14	0.50	1.03	0.47	0.63	0.88±0.65
LA 1000	0.70	0.59	1.69	1.40	1.68	0.73	1.13±0.51
LA 5000	0.48	1.18	0.77	0.98	1.99	1.81	1.20±0.59
LA 15000	0.29	1.08	0.62	0.87	0.47	0.45	0.63±0.29

All data shown on tables 1 and 2 were combined and analyzed. The combined results are shown on table 3 and figure 1.

Table 3: Combined results of the effect of LA 100 - LA 15000 on bone resorption

Group	number	Mean ± SD
Baseline	12	1.00±0.44
Control	12	0.00±0.00(***)
LA 100	12	0.74±0.20
LA 500	12	0.88±0.48
LA 1000	12	1.15±0.40
LA 5000	12	1.27±0.63
LA 15000	12	0.84±0.59

Osteoclast differentiation assay

In the osteoclast differentiation assay, the amount of TRAP 5b activity released into the culture medium was determined as an index of osteoclast number. The results are shown as relative TRAP Sb activities obtained by dividing each individual TRAP 5b activity with the mean TRAP 5b activity of the baseline group.

Table 4: Relative TRAP 5b activities in the first osteoclast differentiation assay

Group	1	2	3	4	5	6	Mean ± SD
Baseline	1.32	0.72	0.43	0.45	1.89	1.18	1.00±0.57
Control	0.16	0.17	0.18	0.11	0.11	0.20	0.16±0.04(**)
LA 100	0.81	0.96	0.43	1.39	0.98	0.65	0.87±0.33
LA 500	0.73	0.55	0.48	0.87	0.58	1.05	0.71±0.22
LA 1000	0.58	0.82	0.35	0.40	0.98	0.45	0.60±0.25
LA 5000	0.44	0.40	0.41	0.36	0.51	0.52	0.44±0.06(*)
LA 15000	0.14	0.26	0.21	0.34	0.31	0.88	0.36±0.27(*)

Table 5: Relative TRAP 5b activities in the second osteoclast differentiation assay

Group	1	2	3	4	5	6	Mean ± SD
Baseline	1.27	1.37	0.98	0.92	0.74	0.71	1.00±0.27
Control	0.17	0.34	0.14	0.10	0.11	0.06	0.15±0.10(***)
LA 100	0.64	0.66	0.62	0.36	0.33	0.62	0.54±0.15(**)
LA 500	1.16	1.30	0.85	1.33	0.76	1.01	1.07±0.24
LA 1000	0.70	0.78	0.34	0.65	0.69	1.00	0.69±0.21
LA 5000	0.94	0.46	0.21	0.72	0.68	0.33	0.56±0.27(*)
LA 15000	0.22	0.31	0.35	0.25	0.15	0.20	0.25±0.07(***)

The assay with LA 100 was repeated one more time, because the results were significantly different from baseline in the second assay, and not significantly different in the first assay.

Table 6: Relative TRAP 5b activities in the third osteoclast differentiation assay with LA 100.

Group	1	2	3	4	5	6	Mean ± SD
Baseline	1.25	1.20	0.76	0.93	1.07	0.81	1.00±0.20
Control	0.08	0.07	0.20	0.10	0.25	0.13	0.14±0.07(***)
LA 100	0.71	0.96	0.42	0.47	0.87	0.69	0.69±0.21(*)

All data shown on tables 4-6 were combined and analyzed. The combined results are shown on table 7 and figure 2.

Table 7: Combined results of the effect of LA 100 - LA1 5000 on osteoclast differentiation

Group	number	Mean ± SD
Baseline	18	1.00±0.36
Control	18	0.15±0.07(***)
LA 100	18	0.70±0.27(**)
LA 500	12	0.89±0.29
LA 1000	12	0.65±0.23(**)
LA 5000	12	0.50±0.20(***)
LA 15000	12	0.30±0.19(***)

Osteoblast differentiation assay

Osteoblast differentiation was determined by measuring cellular alkaline phosphatase (ALP) activities and total protein amounts from cell lysates. The ALP activities were divided with the corresponding protein amounts to obtain specific activities of ALP. The results are shown as relative specific activities obtained by dividing each individual value with the mean value of the baseline group.

Table 8: Relative specific activities of intracellular alkaline phosphatase in the preliminary osteoblast differentiation assay

Group	1	2	3	4	Mean ± SD
Baseline	0.94	1.10	0.94	1.02	1.00±0.07
Control	1.10	1.32	1.31	1.29	1.26±0.10(**)
LA 100	0.98	1.29	1.19	1.12	1.15±0.13
LA 500	0.96	0.98	0.99	1.11	1.01±0.07
LA 1000	0.69	1.13	0.92	1.01	0.94±0.19
LA 5000	0.42	0.46	0.50	0.48	0.47±0.03(***)
LA 15000	0.51	0.49	0.47	0.54	0.50±0.03(***)

Table 9: Relative specific activities of intracellular alkaline phosphatase in the first osteoblast differentiation assay

Group	1	2	3	4	5	6	7	8	Mean ± SD
Baseline	0.97	0.94	1.12	0.98	0.97	1.06	0.99	0.96	1.00±0.06
Control	1.01	1.20	1.04	1.13	1.19	1.06	1.03	1.14	1.10±0.08(**)
LA 100	1.25	0.98	1.31	0.77	0.95	1.04	1.13	0.98	1.05±0.17
LA 500	0.83	1.03	1.02	0.98	0.95	0.96	0.82	0.62	0.90±0.14
LA 1000	1.01	1.12	1.06	0.76	1.01	0.78	0.93	0.81	0.94±0.14
LA 5000	0.54	0.48	0.47	0.63	0.54	0.59	0.44	0.55	0.53±0.06(***)
LA 15000	0.40	0.42	0.53	0.36	0.39	0.35	0.30	0.43	0.40±0.07(***)

Table 10: Relative specific activities of intracellular alkaline phosphatase in the second osteoblast differentiation assay

Group	1	2	3	4	5	6	Mean ± SD
Baseline	0.99	0.83	1.25	1.01	0.88	1.04	1.00±0.15
Control	1.00	1.18	1.53	1.52	1.03	1.38	1.27±0.24(*)
LA 100	0.91	0.94	1.34	1.20	1.00	1.43	1.14±0.22
LA 500	0.88	0.89	1.10	1.09	0.75	0.90	0.93±0.14
LA 1000	0.73	0.71	1.19	0.81	0.72	1.09	0.88±0.21
LA 5000	0.31	0.51	0.51	0.49	0.28	0.40	0.41±0.10(***)
LA 15000	0.27	0.13	0.33	0.32	0.29	0.31	0.28±0.07(***)

All data shown on tables 8-10 were combined and analyzed. The combined results are shown on table 11 and figure 3.

Table 11: Combined results of the effect of LA100-LA15000 on osteoblast differentiation

Group	number	Mean ± SD
Baseline	18	1.00±0.09
Control	18	1.19±0.17(***)
LA 100	18	1.10±0.18(*)
LA 500	18	0.94±0.13
LA 1000	18	0.92±0.17
LA 5000	18	0.48±0.09(***)
LA 15000	18	0.38±0.11(***)

Bone formation assay

The amount of bone formation was determined by measuring the amount of calcium deposited into bone nodules formed by mature osteoblasts. The results are shown as the amount of calcium released (mmol/L) from the bone nodules after HCl extraction. The baseline values are too low to show the results using relative amounts as was done in the other assays.

Table 12: Calcium deposition (mmol/L) in the preliminary bone formation assay

Group	1	2	3	4	Mean ± SD
Baseline	0	0	0	0	0.00±0.00
Control	0.04	0	0	0.04	0.02±0.02
LA 100	0	0	0	0	0.00±0.00
LA 500	0	0	0	0.09	0.02±0.05
LA 1000	0.10	0	0.11	0.05	0.07±0.05(*)
LA 5000	0.59	1.64	0.39	1.62	1.06±0.66(***)
LA 15000	1.48	0.16	0.50	1.41	0.89±0.66(***)

Table 13: Calcium deposition (mmol/L) in the first bone formation assay

Group	1	2	3	4	5	6	Mean ± SD
Baseline	0	0	0	0.02	0.02	0	0.01±0.01
Control	0.15	0.21	0.14	0.10	0.15	0.16	0.15±0.04(***)
LA 100	0.04	0.17	0.01	0.27	0	0.14	0.11±0.11(*)
LA 500	0.44	0.15	1.32	0.27	1.31	1.10	0.77±0.54(***)
LA 1000	0.95	1.66	1.47	1.41	1.00	1.25	1.29±0.28(***)
LA 5000	1.31	1.55	1.56	1.52	1.40	1.39	1.46±0.10(***)
LA 15000	1.46	1.42	1.56	1.11	1.11	1.08	1.29±0.21(***)

Table 14: Calcium deposition (mmol/L) in the second bone formation assay

Group	1	2	3	4	5	6	7	8	Mean ± SD
Baseline	0	0.01	0	0.01	0	0	0.02	0	0.01±0.01
Control	0.22	0.14	0.16	0	0.16	0	0.10	0.16	0.12±0.08(**)
LA 100	0.04	0.18	0	0	0	0.28	0.14	0	0.08±0.11
LA 500	0.17	0.30	1.41	0	0.02	0.46	1.17	1.40	0.62±0.61 (*)
LA 1000	1.09	0.81	1.34	1.56	1.76	0.02	1.52	1.02	1.14±0.55(***)
LA 5000	1.70	1.44	1.64	1.52	1.08	1.63	1.30	1.48	1.47±0.20(***)
LA 15000	1.24	1.46	1.22	1.68	1.62	1.18	1.21	1.56	1.40±0.21(***)

The data shown on tables 13 and 14 were combined and analyzed. The results from table 12 were not included as there was no significant difference between the baseline and the control groups. The combined results are shown on table 15 and figure 4.

Table 15: Combined results of the effects of LA 100 -LA 15000 on bone formation activity of mature osteoblasts

Group	number	Mean ± SD
Baseline	14	0.01±0.01
Control	14	0.13±0.06(***)
LA 100	14	0.09±0.10(**)
LA 500	14	0.68±0.56(***)
LA 1000	14	1.20±0.45(***)
LA 5000	14	1.47±0.16(***)
LA 15,000	14	1.35±0.21(***)

ANALYSIS OF THE DATA

Bone resorption assay

In the bone resorption assay, there was no significant effect of the lanthanum (III) ion on either the amount of CrossLaps released into the culture medium or on the osteoclast number. The control substance, bafilomycin A1, completely inhibited bone resorption. As shown on table 3 and figure 4, the lanthanum (III) ion has no statistically significant effects on the bone resorbing activity of individual mature osteoclasts at any of the concentrations tested. However, the dose-dependent inhibition of bone resorption with the lower concentrations (LA 100 and LA 500) should be noticed. The slight decrease seen with LA 15000 may be due to slight toxic effects of this high concentration.

Osteoclast differentiation assay

In the osteoclast differentiation assay, a clear dose-dependent inhibition was observed with LA 500 - LA 15000 that was statistically significant from LA 1000 to LA 15000. A statistically significant inhibition was also

observed with LA 100. In the control group where vitamin D was omitted, osteoclast differentiation was significantly lower than in the baseline group.

Osteoblast differentiation assay

The lanthanum (III) ion showed a clear dose-dependent response in the osteoblast differentiation assay. The highest test concentrations (LA 5000 and LA 15000) inhibited, and the lowest test concentration (LA 100) activated osteoblast differentiation significantly. No significant response was observed with LA 500 and LA 1000. The control substance, 17β-estradiol, activated osteoblast differentiation significantly.

Bone formation assay

All concentrations of the lanthanum (III) ion tested showed a highly significant activation of the bone formation activity of mature osteoblasts, the activation being highest with the highest test concentrations. The control substance, 17β-estradiol, activated bone formation significantly.

SUMMARY

The effects of the test concentrations of the lanthanum (III) ion on the activity and differentiation of bone cells are summarized on table 17, where (+) means significant activation, (−) significant inhibition, and (0) no effect. One character (+ or -) means a p-value between 0.05 and 0.01, two characters (++ or --) a p-value between 0.01 and 0.001, and three characters (+++ or ---) a p-value <0.001.

Table 17: The effects of LA on bone cells

Dose, ng/ml	Bone resorption	Osteoclast differentiation	Osteoblast differentiation	Bone formation
100	0	--	+	++
500	0	0	0	+++
1000	0	--	0	+++
5000	0	---	---	+++
15000	0	---	---	+++

CONCLUSIONS

- The lanthanum (III) ion is a powerful stimulator of the bone formation activity of mature osteoblasts at all concentrations tested, the best responses observed with the highest test concentrations (LA 5000 and LA 15000). However, these concentrations may also have cytotoxic effects on the osteoblast precursor cells, which may compensate the activation of mature osteoblasts *in vivo*.

- LA 500 and LA 1000 also stimulate bone formation, but these concentrations do not decrease the formulation of osteoblasts in the osteoblast differentiation assay, suggesting that they have no cytotoxic effects on osteoblast precursor cells. However, LA 1000 decreases the formation of osteoclasts in osteoclast differentiation assay, suggesting that it may have cytotoxic effects on osteoclast precursor cells. The only significant effect of LA 500 in the four assays was the activation of bone formation. Thus, this concentration of LA may be useful in increasing the bone formation without cytotoxic effects.

- LA 100 appears to activate both bone formation and osteoblast differentiation, and inhibit osteoclast differentiation and bone resorption (although the

inhibition of bone resorption is not statistically significant). All these effects would strengthen bones.

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Fig. 1: Combined results of the effect of LA on bone resorption

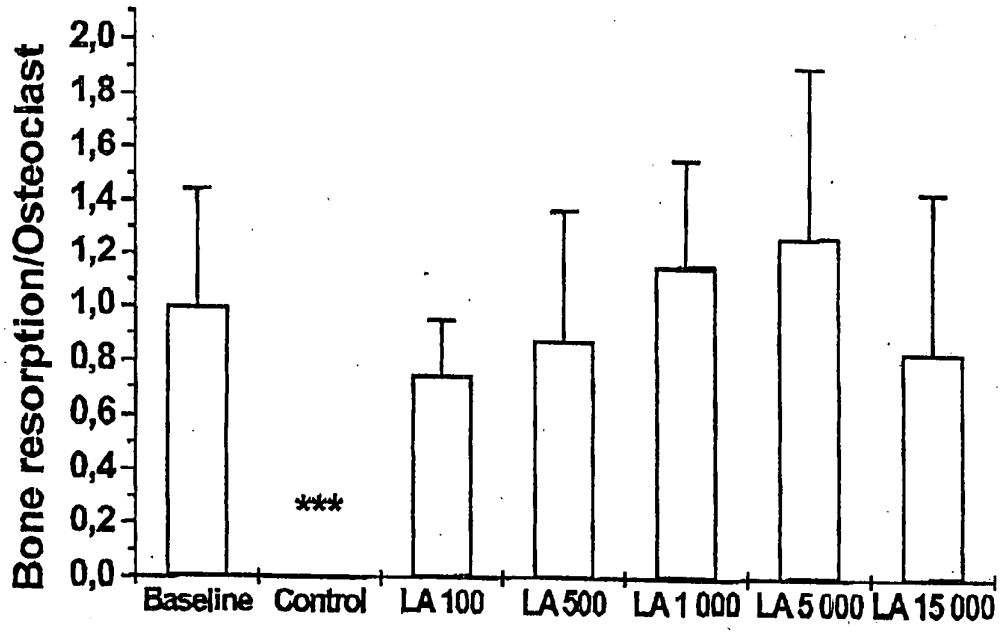

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Fig. 2: Combined results of the effect of LA on osteoclast differentiation

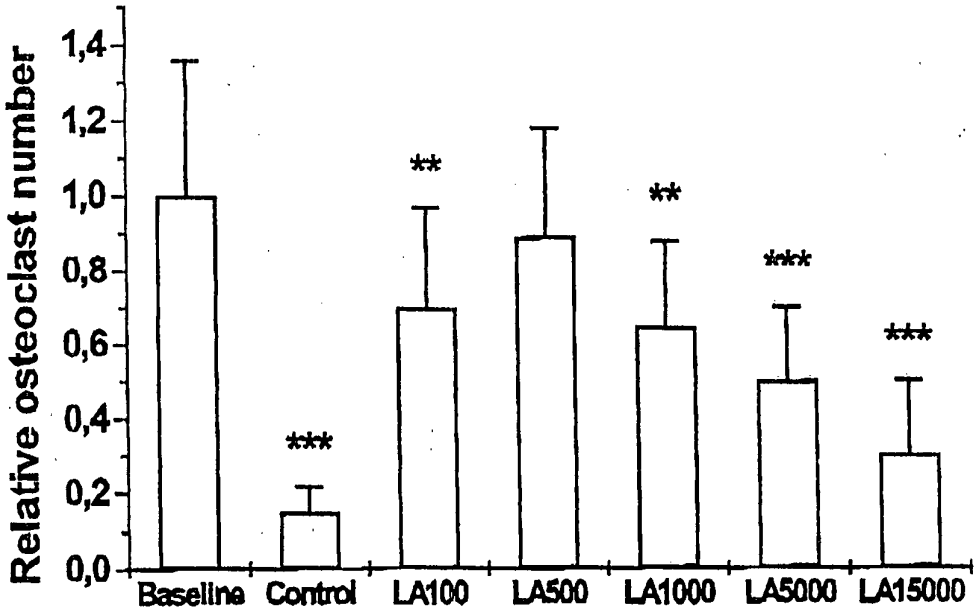

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Fig. 3: Combined results of the effect of LA on osteoblast differentiation

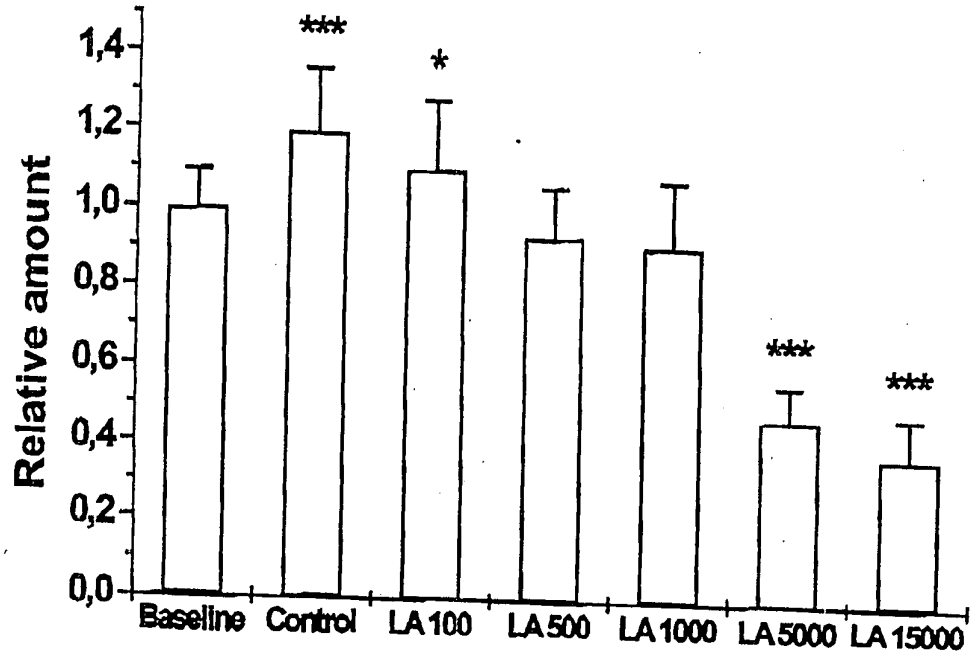

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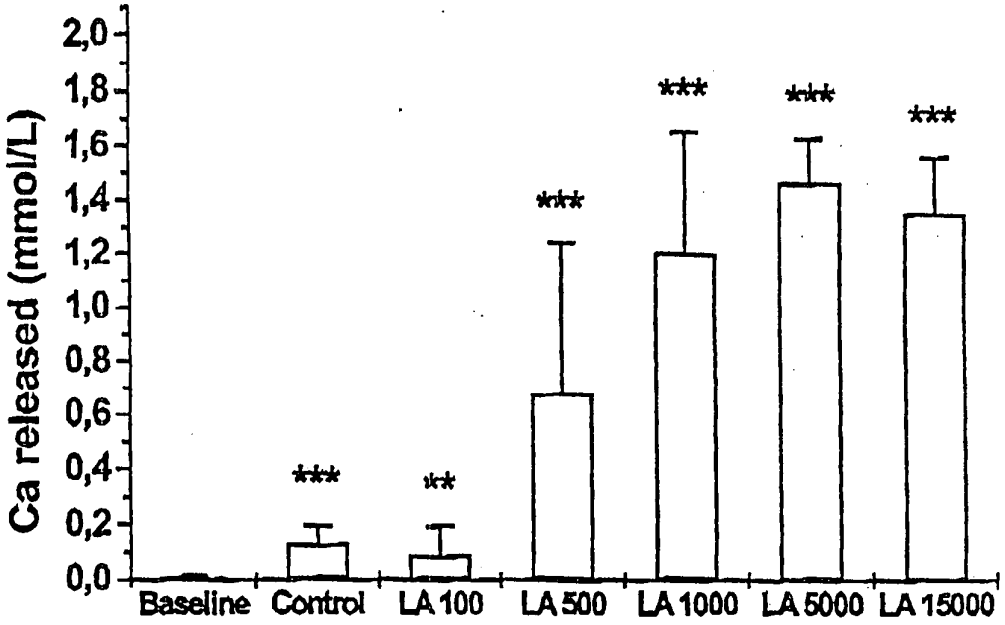
Fig. 4: Combined results of the effect of LA on bone formation activity of mature osteoblasts



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